PB82-236506

The Effect of SO2 on the Uptake of Particles by Mouse Bronchial Epithelium

Harvard School of Public Health
Boston, MA

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

1980

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/J-80-395	2. JOURNAL ARTICLE
3. RECIP	PB82-236506
4. TITLE AND SUBTITLE: The Effect of SO_2 on the Uptake of Particles by Mouse Bronchial Epithelium	5. REPORT DATE
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): Ann Y. Watson and Joseph D. Brain	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Department of Physiology, Harvard University School of Public Health, 665 Huntington Ave., Boston, MA 02115	10. PROGRAM ELEMENT NO. C9GA1A
	11. CONTRACT/GRANT NO. Grant No: R805091
12. SPONSORING AGENCY NAME AND ADDRESS: Office of Research and Development, Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES: Reference: Experimental Lung Research, 1:67-87, 1980	

16. ABSTRACT

In three experiments, we have explored the uptake and transport of collidal gold (Au) and iron oxide (Fe_2O_3) by normal and SO_2-injured bronchial epithelium. In the first experiment mice were exposed to a 2-hr aerosol of Au; in the second experiment, mice were exposed to SO_2 (250 ppm) for 3 hr, then to Au; and in the third experiment, mice were exposed to SO_2 and days postexposure. From each animal, samples of large airways were examined by electron microsopy, and the cell type and particle location were recorded. Au was not readily taken up by the bronchial epithelium in that only two cells out of 1162 contained particles. No Au was seen in the submucosa. Exposure to SO_2 produced a mild, but nonnecrotic response chatacterized by an increase in mucus, vocuolated cells, detached cilia, and an influx of polymorphonuclear leukocytes. SO_2 did not affect the uptake of Au; 2 out of 1177 epithelial cells contained Au. However, gold did reach the submucosa. Results from the SO_2 plus Fe_2O_3 experiment were compared to a previous study of similar design in which mice were exposed only to Fe_2O_3. SO_2 increased the uptake of iron and affected the distrubution of intracellulat iron among the different cell types. It is possible that increased iron uptake is due to either injury or to changes in particle solubility produced by the SO_2.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY NATIONAL TECHNICAL INFORMATION SERVICE U.S. DEPARTMENT OF COMMERCE SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT: Release to Public	19. SECURITY CLASS (This Report): Unclassified	21. NO. OF PAGES
	20. SECURITY CLASS (This page): Unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

EPA-600/J-80-395
JOURNAL ARTICLE

The Effect of SO_2 on the Uptake of Particles by Mouse Bronchial Epithelium

Ann Y. Watson and Joseph D. Brain

ABSTRACT: *In three experiments, we have explored the uptake and transport of colloidal gold (Au) and iron oxide (Fe_2O_3) by normal and SO_2-injured bronchial epithelium. In the first experiment, mice were exposed to a 2-hr aerosol of Au; in the second experiment, mice were exposed to SO_2 (250 ppm) for 3 hr, then to Au; and in the third experiment, mice inhaled SO_2 and then were exposed to Fe_2O_3. In each experiment, animals were killed at 0 hr, 1 day, and 4 days postexposure. From each animal, samples of large airways were examined by electron microscopy, and the cell type and particle location were recorded. Au was not readily taken up by the bronchial epithelium in that only two cells out of 1162 contained particles. No Au was seen in the submucosa. Exposure to SO_2 produced a mild, but nonnecrotic response characterized by an increase in mucus, vacuolated cells, detached cilia, and an influx of polymorphonuclear leukocytes. SO_2 did not affect the uptake of Au; 2 out of 1177 epithelial cells contained Au. However, gold did reach the submucosa. Results from the SO_2 plus Fe_2O_3 experiment were compared to a previous study of similar design in which mice were exposed only to Fe_2O_3. SO_2 increased the uptake of iron and affected the distribution of intracellular iron among the different cell types. It is possible that increased iron uptake is due to either injury or to changes in particle solubility produced by the SO_2.*

INTRODUCTION

The uptake and transport of particulate material from the lumen of airways have been studied in a number of recent investigations. Horseradish peroxidase [1, 2], asbestos [3], carbon particles [4], ferritin [5], and iron oxide [6] have been shown to be taken up by airway epithelia in either in vitro or in vivo studies. When particles are taken up by epithelial cells and/or have access to the underlying connective tissue, the opportunity exists for cancer induction, immunological responses, or other alterations depending on the nature of the particle. Such opportunities might be greatly increased if the normal integrity of the airway epithelium is compromised. There is evidence that if epithelial tight junctions are disrupted [7] or if the epithelium is discontinuous [6] then penetration of material to the submucosa is greater. It is, therefore, of interest to assess the extent of particle transport in injured epithelial tissues.

We elected to produce mild injury by sulfur dioxide (SO_2) inhalation; SO_2 is a highly water-soluble gas and is quickly removed by the upper respiratory tract [8]. Therefore, it exerts most of its influence in the trachea and upper airways.

From the Department of Physiology, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115.

Correspondence and requests for reprints to: Ann Y. Watson, Department of Anatomy, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115.

Received 11 August 1979; accepted 13 October 1979.

0190-2148/80/010067–21$02.25

Airways of animals chronically exposed to SO_2 demonstrate an increase in mucous production, an influx of polymorphonuclear leukocytes, loss of cilia, and vacuolated cells. If the dose is high or prolonged enough, sloughing of epithelial cells with subsequent hyperplasia also occurs [9–11]. It should be noted that SO_2 was used as an agent to produce airway injury, and the purpose of this study was not to evluate SO_2 as an atmospheric pollutant.

In a previous study [6], we observed that as many as 50% of the epithelial cells in normal mouse airways accumulated iron compounds following exposure to an iron oxide aerosol. In this report, we describe a parallel study of particle uptake using colloidal gold. Colloidal gold and iron oxide were chosen because they are nontoxic, readily distinguished by electron microscopy, and are easily generated as a submicrometric aerosol. We also describe another series of experiments in which mice breathed SO_2 and were then exposed to aerosols of colloidal gold or iron oxide. Our purpose was to quantify the extent of particle uptake in injured airway epithelium and compare it to that seen in the normal airway.

MATERIALS AND METHODS

Animals and Experimental Design

Male mice (CD-1 strain, Charles River, Wilmington, MA) weighing 20–25 g were used in this four-part study. In the first three experiments there were nine animals per experiment. In each study, three animals were sacrificed immediately after an aerosol exposure, three at 1 day, and three at 4 days postexposure. In the first experiment, spontaneously breathing animals were exposed to an aerosol of colloidal gold (180 mg/m^3) for a 2-hr period. In the second experiment, mice were exposed first to 250 ppm of SO_2 for 3 hr; then 30 min later, they were exposed to the colloidal gold aerosol (152 mg/m^3) for 2 hr. In the third experiment, mice were again exposed to 250 ppm of SO_2 for 3 hr; after 30 min they were exposed to an iron oxide aerosol (294 mg/m^3) for 2½ hours. Finally, in order to determine whether SO_2 exposure influenced particle retention (ventilation × collection efficiency), we studied two groups of animals. One group was exposed to SO_2 at 250 ppm for 3 hr. At 30 min postexposure, those mice plus a second group of non-SO_2 exposed mice breathed a ^{99m}Tc-labeled sulfur colloid aerosol (mass concentration, 72 μg/l; activity concentration, 54 μC/l) for 2¼ hours. All 10 animals were killed immediately after the exposure. A summary of the experimental design is shown in Table 1.

Atmospheres Generated

SO_2 was obtained from a cylinder of 100% SO_2 (Curtis Matheson). The desired concentration was obtained by adjusting the relative flows from the cylinder and a source of filtered dilution air. Approximately 30 air changes per hour were maintained in the exposure chamber. The concentration was monitored by conductivity and flame photometry techniques. Samples were withdrawn from the exposure chamber through a midget impinger containing hydrogen peroxide, a trapping reagent. The conductivity of the resulting solution was measured with an electrode and the sample values compared to a calibration curve. A Meloy Model SA 285 FPD Sulfur Analyzer was used for the flame photometric method. This instrument performs continuous analysis of sulfur compounds in gas mixtures. A Meloy SO_2 calibration source was used to calibrate the analyzer. All experiments were conducted at about 250 ppm, and readings were taken every 15 or 30 min and time-weighted averages (TWA) computed. For the SO_2 plus Au

Table 1 Summary of experimental protocol

Experiment Number	Number of Animals	Exposure Atmosphere	Sacrifice Time Points
1	9	Au	0 hr, 1 day, 4 day
2	9	SO_2 + Au	0 hr, 1 day, 4 day
3	9	SO_2 + Fe_2O_3	0 hr, 1 day, 4 day
4	5	^{99m}Tc	0 hr
	5	SO_2 + ^{99m}Tc	0 hr

experiment, TWA = 248 ± 11 ppm; for the SO_2 plus Fe_2O_3 experiment, TWA = 254 ± 8 ppm.

To make the colloidal gold suspension, 10 ml of chlorauric acid (20 mg/ml, pH 6–7) were heated to 68°C in a shaker bath. To the heated chlorauric acid, 4 ml of sodium acetate (100 mg/ml) and 3 ml of gelatin (40 mg/ml) were added slowly. The temperature of the suspension was raised to 70°C and 2 ml of ascorbic acid (100 mg/ml) was rapidly added while stirring. Finally, 1 ml of sodium citrate (80 mg/ml) was added. The final gold-solid concentration in the 20 ml of solution was 5 mg/ml. The colloidal gold suspension was sonicated for 1 hr prior to use and was aerosolized using a 5-MHz ultrasonic nebulizer (Charles Stark Draper Laboratory, MIT, Cambridge, MA). Samples of the aerosol were taken by electrostatic precipitation and viewed in a Phillips 300 electron microscope. The electron-dense aerosol appeared as polydisperse submicrometric agglomerates. Easily distinguished gold particles that made up the aerosol were approximately 300 A° in diameter (see Fig. 2).

The iron oxide aerosol was generated by the combustion of iron pentacarbonyl vapors in a heated furnace. Details of the iron oxide generator and its operation are described elsewhere [12]. The aerosol used has a mass median aerodynamic diameter of 0.15 μm and a geometric standard deviation of 2.2. This aerosol is a loose lacy agglomerate of smaller spherical iron oxide particles about 0.005 μm in diameter. These agglomerates have a characteristic appearance and are readily distinguished by electron microscopy [13].

The ^{99m}Tc-labeled sulfur colloid was prepared using a standard Mallinckrodt TechneColl kit (Mallinckrodt Nuclear, St. Louis, MO). The aerosol was generated with the same 5-MHz ultrasonic nebulizer. The aerosol produced has been sized on a concentric aerosol centrifuge and its aerodynamic diameter distribution forms a log-normal curve with an activity median aerodynamic diameter of 0.38 μm and a geometric standard deviation of 1.35.

Tissue Preparation

In the first three studies, the mice received an intraperitoneal injection of heparin prior to death to ensure adequate postmortem perfusion of the pulmonary circulation. After 2 min, they were anesthetized with an intraperitoneal injection of pentobarbital. The trachea was exposed and cannulated. The animal was exsanguinated by cutting the renal artery. The lungs were collapsed by snipping the diaphragm, and the rib cage was opened. The lungs were inflated to 30 cm H_2O (Total lung capacity, TLC), deflated to 12 cm H_2O (50% TLC), and then maintained at that pressure during fixation.

The lungs were fixed in situ by perfusion via the pulmonary arteries at a pressure of 60 mm Hg. Following a 2-min perfusion with a phosphate-buffered saline and heparin (5 U/ml) solution with an osmolarity of 350 mOsm, the lungs were

perfused for an additional 10 min with a modified Karnovsky's fixative [14]. The modification entailed the dilution of the cacodylate buffer to 0.05 M to achieve an osmolarity of approximately 950–1000 mOsm. The final formaldehyde concentration was 2.0% and the glutaraldehyde concentration was 2.5%. The lungs were then excised, separated into lobes, and kept in fixative at 4°C overnight. The tissue was postfixed in 1% OsO_4 (aq) for 2 hr on ice. For lungs from animals exposed to iron oxide, the osmium solution was reduced with 1.5% potassium ferrocyanide [15]. The tissue was dehydrated through an ethanol series and embedded in Epon.

In an experiment using a radiolabeled aerosol of colloidal gold, the fixative used in the overnight immersion was saved and the radioactivity counted. This was done in order to determine whether gold particles had been washed from the lung tissue. Less than 0.1% of the deposited gold was found in the fixative solution.

From each animal, four blocks containing large intrapulmonary bronchi were selected for analysis. Thin sections for electron microscopy were collected on Formvar-coated 75-mesh copper grids. Sections from animals exposed to colloidal gold were stained with uranyl acetate and lead citrate; whereas sections derived from animals exposed to iron oxide were stained with the bismuth subnitrate stain for ferritin [16]. Using a Phillips 300 electron microscope, one section from each block was examined for a total of 4 sections per animal and 36 sections per experiment. By using 75-mesh grids (77% open area) usually one section could be found that did not fall upon any grid bars. All cells comprising the epithelium were examined and classified as ciliated, nonciliated, basal, or unknown and the presence of gold particles or iron compounds on the surface, within cells or underneath cells was noted.

In the final study dealing with the effect of SO_2 on particle retention, the animals were killed immediately after breathing the ^{99m}Tc aerosol. The lungs and trachea were excised, weighed, and counted in a gamma well counter.

RESULTS

Colloidal Gold Alone

Table 2 presents the distribution of gold particles in relation to the different cell classes (ciliated, nonciliated, basal or unknown) at each of the three time points studied. We were interested in the amount of gold present on cell surfaces immediately after the exposure. Since the animals were killed serially, we were able to quantitate gold distribution during the first 2 hr after the aerosol exposure. Figure 1 shows a decrease in the amount of gold on cell surfaces over time in animals exposed to either colloidal gold or colloidal gold plus SO_2. The times shown represent the interval between the end of the aerosol exposure and the point at which the fixation by perfusion was begun. Figure 2 illustrates the appearance of the colloidal gold on the surface of a ciliated cell from an animal fixed 15 min after removal from the aerosol chamber. The gold particles appear to be evenly distributed throughout the mucous layer and were often seen in close proximity to cell surfaces. There was never any indication that the gold particles had been translocated during the preparation or sectioning of the tissue. Particles were never observed in inappropriate intracellular sites such as cilia, nuclei, or mitochondria. Only one epithelial cell, a ciliated cell, contained any gold and this particle was located in a multivesicular body (Fig. 3). In the

Table 2 Location of gold particles as a function of time, cell class and SO_2 exposure

Time Postexposure	Location No SO_2 (SO_2)	Cell class: Ciliated		Nonciliated		Basal		Unknown		Total cells	
		No SO_2	(SO_2)	No SO_2	(SO_2)	No SO_2	(SO_2)	No SO_2	(SO_2)	No SO_2	(SO_2)
0 hr	Cell surface	87	(74)	67	(84)	–	(–)	1	(3)	155	(161)
	Within cells	1	(0)	0	(0)	0	(0)	0	(0)	1	(0)
	Total examined	161	(145)	171	(230)	10	(11)	2	(12)	344	(398)
1 day	Cell surface	0	(0)	0	(1)	–	(–)	0	(0)	0	(1)
	Within cells	0	(0)	0	(0)	0	(0)	1	(0)	0	(0)
	Total examined	183	(132)	213	(226)	21	(11)	6	(8)	423	(377)
4 day	Cell surface	0	(0)	0	(0)	–	(–)	0	(0)	(0)	(0)
	Within cells	0	(1)	0	(1)	0	(0)	0	(0)	0	(2)
	Total examined	168	(152)	208	(231)	14	(13)	5	(6)	395	(402)

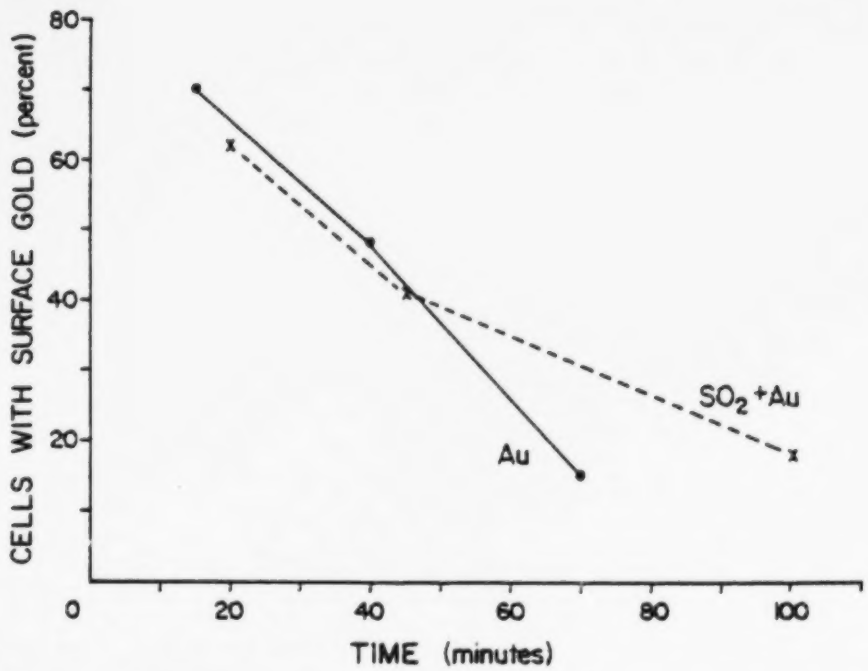

Figure 1 The percentage of cell sections with gold particles above the cell surface is plotted against time postexposure. Each point represents a single animal. Gold experiments with and without SO_2 are illustrated.

underlying connective tissue, no gold particles were seen in fibroblasts, macrophages, ground substance, or smooth muscle cells. By 1 day and 4 days postexposure, no extracellular gold was present. Particles were seen only within airway macrophages with the exception of one epithelial cell (cell type unknown) that contained some gold particles in a dense body (presumptive secondary lysosome) at 1 day. Again, no gold particles were observed in the submucosa.

Colloidal Gold and SO_2

Over half the sections from the 0-hr animals displayed evidence of an epithelial response to the SO_2. Many cells contained large vacuoles that were either empty or contained electron-dense amorphous material. Figure 4(a) shows a comparatively normal nonciliated cell with a large intracellular inclusion. The mucous layer on the surface of this cell was the usual thickness seen on nonciliated cell surfaces in animals not exposed to SO_2. However, many sections from SO_2-exposed animals had an increase in the thickness of the mucous layer [Fig. 4(b)]. Occasionally detached cilia were observed on or within the mucus. Also suggesting an inflammatory response to SO_2, was an influx of polymorphonuclear leukocytes onto luminal surfaces; Fig. 4(c) illustrates a neutrophil on the surface of a ciliated cell. Leukocytes were also seen in the submucosa and between epithelial cells, but epithelial tight junctions appeared intact. There was no indication of sloughing of epithelial cells nor were bare patches of basement membrane observed. We did observe increased numbers of cells undergoing ciliogenesis in the 4-day group. With this exception, the epithelium appeared normal at 1 day

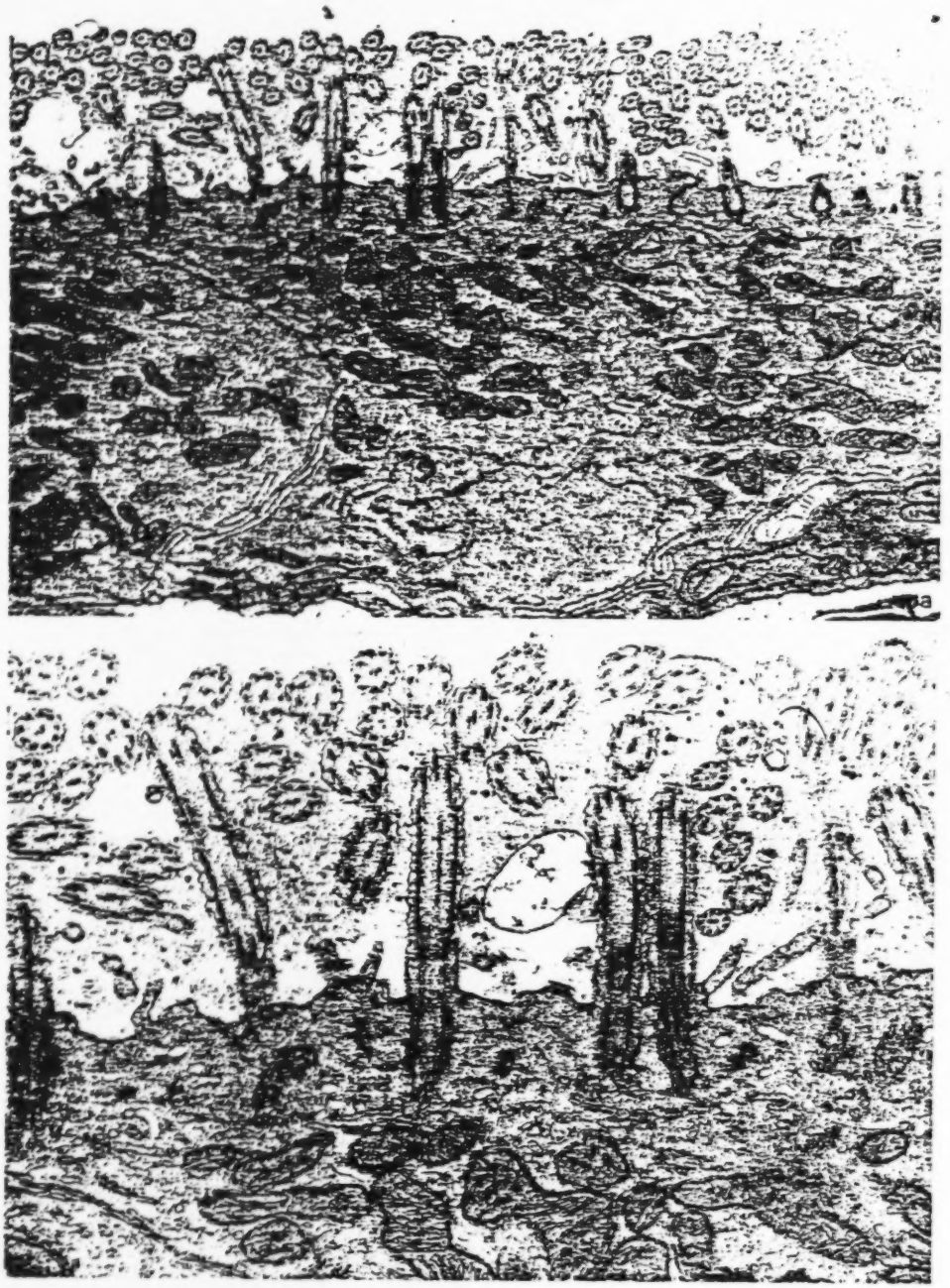

Figure 2 (a). Electron micrograph of colloidal gold on airway surfaces from an animal killed 15 min after the gold aerosol exposure (Uranyl acetate and lead citrate; × 10,775). (b). Higher magnification of the cell surface showing the gold particles within the mucus. Note that a number of particles are close to but not attached to the cell surface (Uranyl acetate and lead citrate; × 24,700).

Figure 3 Electron micrograph of a ciliated cell from an animal killed 15 min after the exposure. Gold particles are present on the cell surface and one intracellular particle can be seen within a multivesicular body (arrow) (Uranyl acetate and lead citrate; × 16,350). Insert: Higher magnification of the multivesicular body with its gold particle (× 50,900).

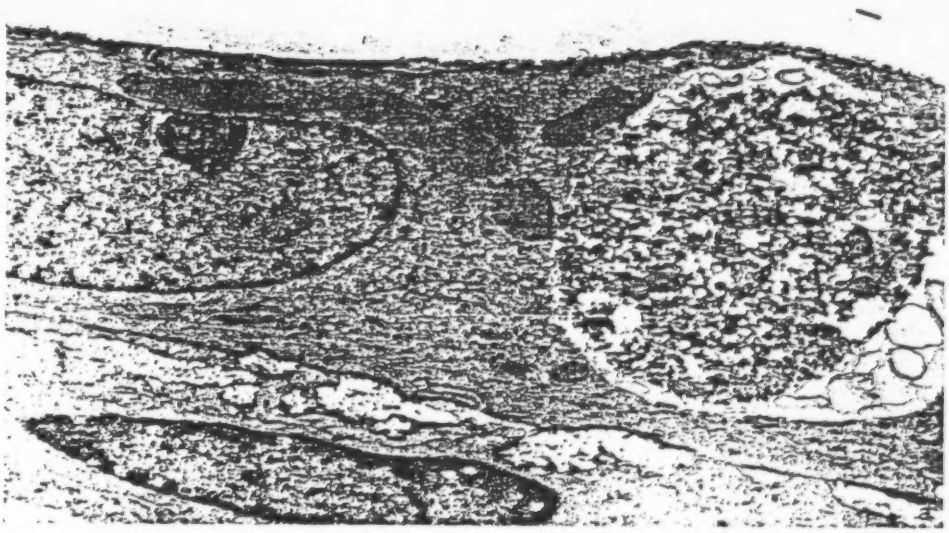

Figure 4 Electron micrograph of the bronchial epithelium in animals exposed to 250 ppm SO_2 for 3 hr. The animals are from the group killed immediately after the subsequent 2-hr colloidal gold exposure. (a). Nonciliated cell with a large vacuole containing amorphous material (Uranyl acetate and lead citrate; × 12,100). (b). Nonciliated cell covered with a mucous layer considerably thicker than that seen in animals not exposed to SO_2 (Uranyl acetate and lead citrate; × 9,050). (c). A polymorphonuclear leukocyte on the surface of a ciliated cell (Uranyl acetate and lead citrate; × 8,900).

and 4 days postexposure. Earlier signs of vacuolation, increased mucus, and airway neutrophils had disappeared.

As before, extracellular gold was observed on airway surfaces immediately after the exposure. Figure 1 illustrates the clearance of free particles from the airway surface over time postexposure. Although gold particles were seen within airway macrophages and leukocytes, no gold particles were seen in airway epithelial cells. Even in those areas where neutrophils were observed migrating onto the lumen, no particles were observed between cells or in the submucosa. By 1 day postexposure, with the exception of a few free particles on airway and alveolar surfaces, any gold present was within macrophages. At 4 days postexposure, however, two epithelial cells (one ciliated and one nonciliated) contained gold particles within dense bodies. Interestingly, this same section revealed four vesicles in connective tissue cells that also contained particles (Fig. 5). Examination of serial sections confirmed that these cells were in the submucosa and were probably fibroblasts. This was the only time that gold particles were observed in the connective tissue compartment in either of the two colloidal gold experiments. Table 2 summarizes the quantitative information on cell class and gold particle location.

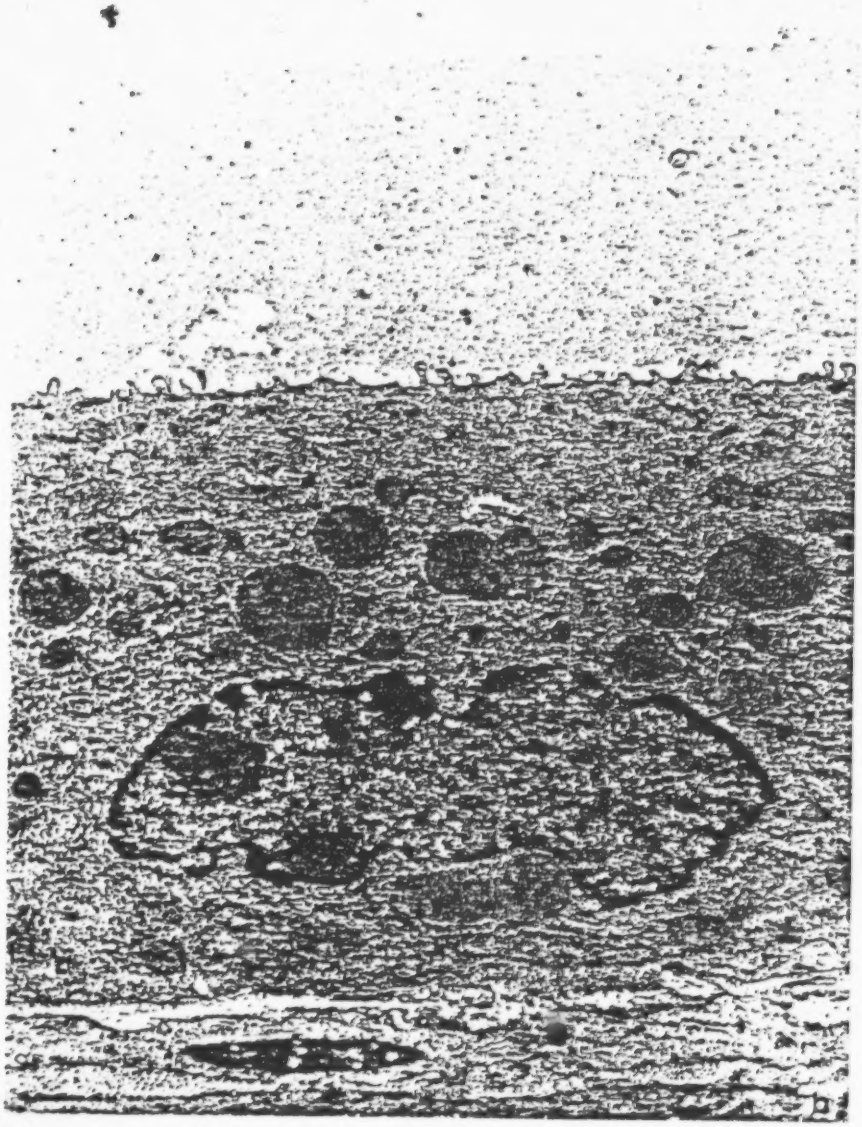

Figure 4 (continued).

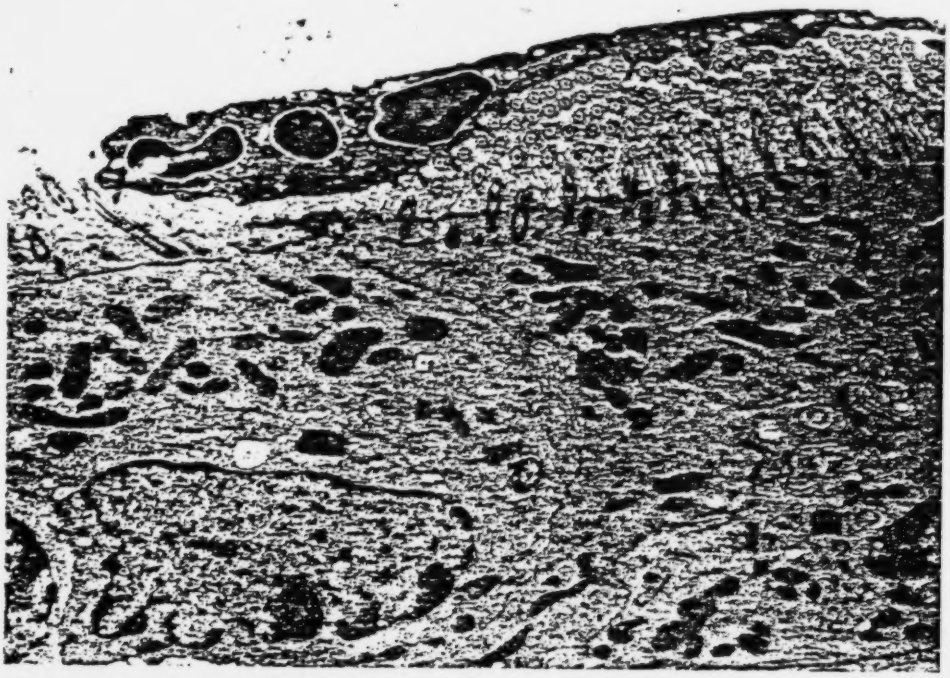

Figure 4 (continued).

Iron Oxide and SO_2

The cellular response to SO_2 was similar to that seen in the experiment just described. Vacuolated cells, increased mucus, detached cilia, and neutrophils were again observed in sections derived from animals sacrificed immediately after the exposure. Figure 6 shows a nonciliated cell with a vacuole containing a mitochondria and other membrane remnants. Mucus of the thickness shown there was not an uncommon finding. A neutrophil can be seen in the mucous layer. Although the level of SO_2 was the same in both experiments, the duration of the response appeared to be longer in this experiment. Many sections from the 1-day group also exhibited these morphological changes, and it was not until 4 days that the airways had returned to normal.

Before discussing the distribution of iron, we need to first describe the various intracellular forms of iron. Upon entry into a cell, iron stimulates the synthesis of ferritin and the formation of hemosiderin which together serve as storage forms for iron. Ferritin is composed of an inorganic core of iron oxyhydroxide and a protein shell termed apoferritin. Hemosiderin results from the proteolytic breakdown of ferritin by the lysosomal system [17]. In our previous study in which mice were exposed to iron oxide without SO_2 [6], iron oxide was seen initially on airway surfaces. In those animals from the 0-hr group, small pinocytic vesicles containing electron-dense material (presumptive iron oxide) were noted in epithelial cells. These vesicles migrated to the Golgi region; later ferritin appeared in Golgi-associated vesicles which fused to form multivesicular bodies and hemosiderin. That paper also discusses the procedures used to identify these iron compounds with certainty [6].

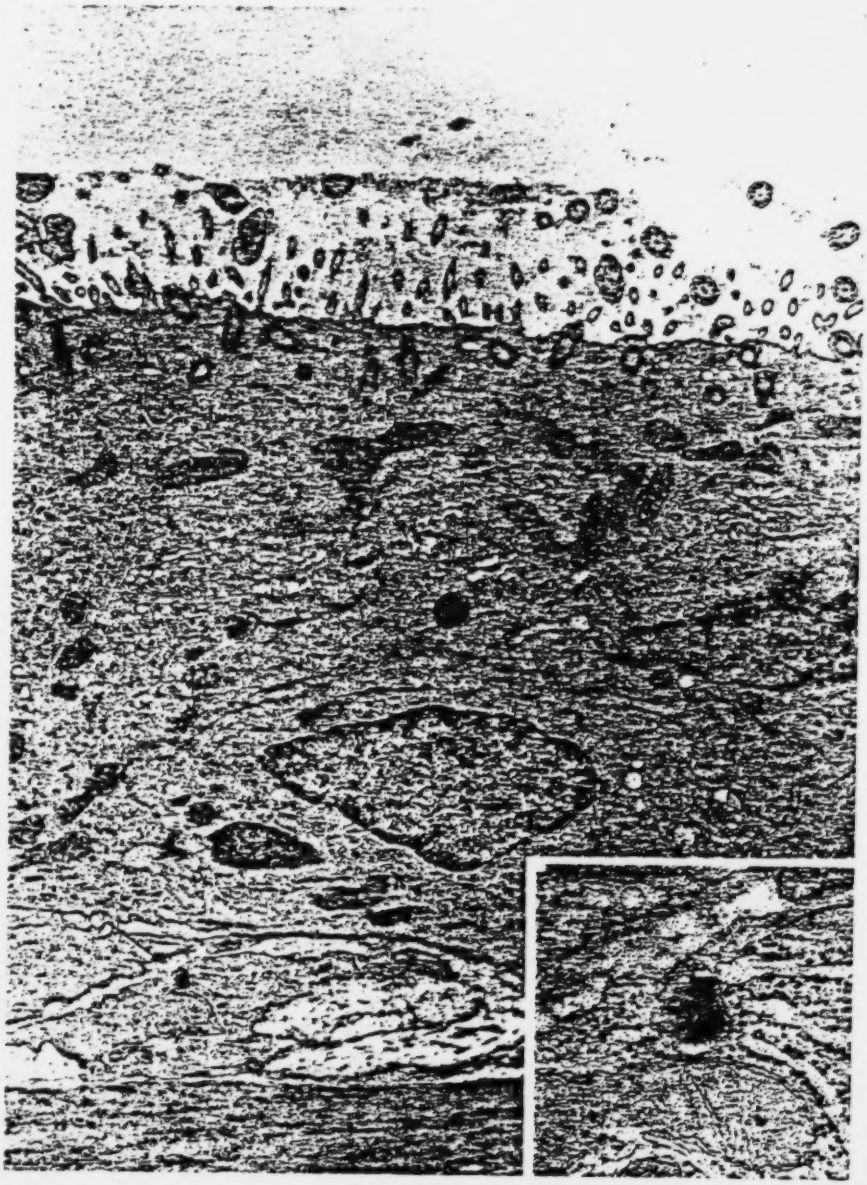

Figure 5 Electron micrograph of an area of epithelium from an SO_2-exposed animal killed 4 days postexposure. Gold particles (arrow) are in a fibroblast in the underlying connective tissue (Uranyl acetate and lead citrate; × 12,950). Inset: Higher magnification of the gold particles within a membrane-bounded vesicle (× 50,900).

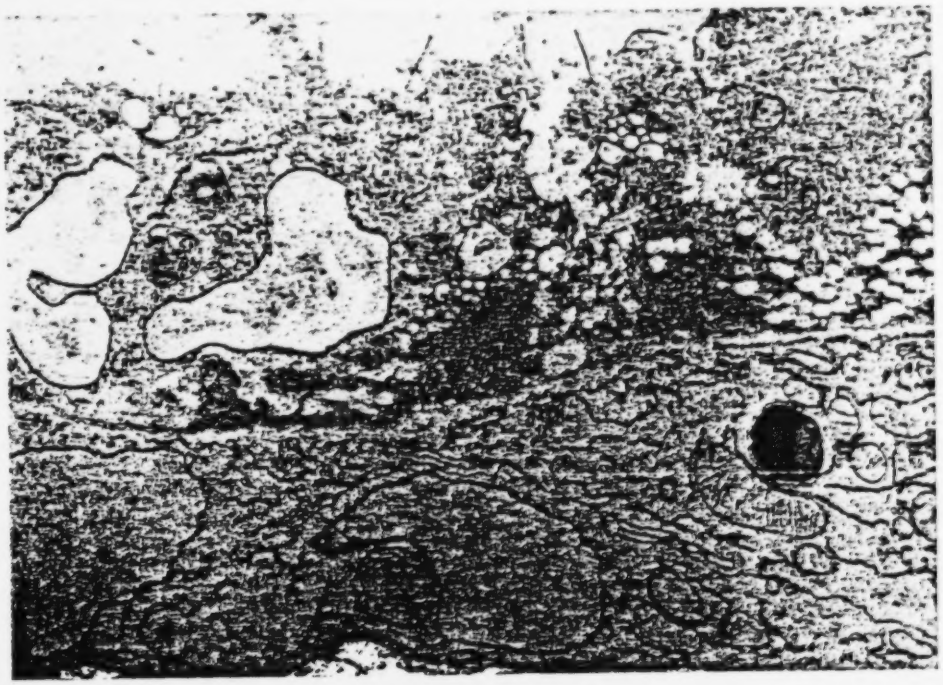

Figure 6 Electron micrograph of a section of airway from an SO_2-exposed animal killed immediately after the Fe_2O_3 exposure. One cell contains an autophagocytic vacuole in the perinuclear region (arrow) and another cell contains an electron-dense hemosiderin granule. The epithelium is covered with a thick mucous layer. Fe_2O_3 and a polymorphonuclear leukocyte can be seen within the mucus. Membranes are detectable because of the use of reduced OsO_4 (Bismuth subnitrate; × 11,100).

In this study, in which mice were exposed to SO_2 prior to a similar iron oxide exposure, the same sequence was observed. Hemosiderin was observed in all cell types. Figure 6 shows a nonciliated cell with an electron-dense hemosiderin granule. Iron oxide can also be seen on the surface within the mucus. Although iron oxide was not seen in the submucosa, hemosiderin was occasionally noted in fibroblasts, macrophages, and smooth muscle cells in the underlying connective tissue (Fig. 7). At 1 day, some free iron oxide was still observed on airway surfaces. As with the 0-hr group, hemosiderin was seen in all cell types of the epithelium and submucosa. By 4 days postexposure, all the iron oxide had been cleared from airway surfaces.

Since we were interested in comparing the results from this experiment with the previous study of iron oxide uptake without SO_2 preexposure, we again chose the presence of hemosiderin as an indication that a cell had taken up iron. If a cell section had one or more hemosiderin granules, it was recorded as being positive. Table 3 summarizes the data obtained. The percentage of hemosiderin-containing cell sections increased over time and the effect of time postexposure was significant at $P < 0.001$. Figure 8 displays this trend of increasing hemosiderin and compares it to the results of our previous study. In the group of animals exposed to SO_2, hemosiderin content is significantly greater at each time point (0 hr, $P < 0.01$; 1 day, $P < 0.001$; 4 days, $P < 0.05$).

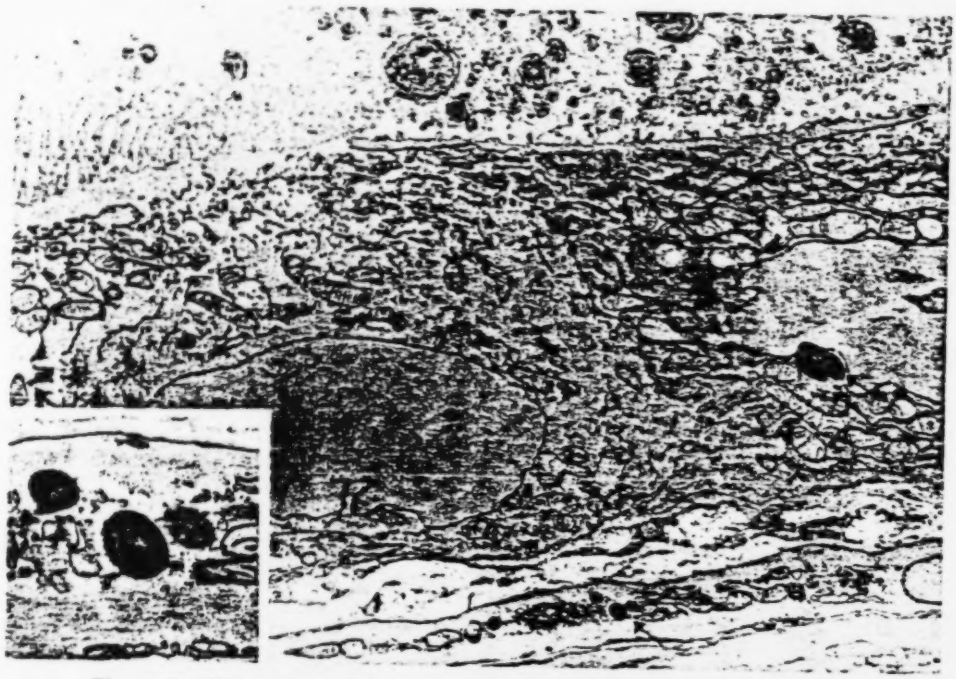

Figure 7 Electron micrograph of a section of epithelium from an SO_2 plus Fe_2O_3-exposed animal killed 1 day postexposure. A thickened mucous layer is still present. Hemosiderin can be seen in a nonciliated cell (double arrow) and a smooth muscle cell (arrow) (Bismuth subnitrate; × 6,900). Insert: Higher magnification of the hemosiderin in the smooth muscle cell (× 25,200).

Immediately after the exposure, there was no difference in the participation of iron uptake between ciliated and nonciliated cells. At 1 day postexposure, nonciliated cells had a higher participation rate ($P < 0.05$). But by 4 days, the ciliated cells had a significantly higher percentage of cell sections with hemosider-

Table 3 Percentage of hemosiderin positive cells after SO_2 exposure as a function of time and cell class

Time postexposure		Cell class				
		Ciliated	Nonciliated	Basal	Unknown	Total cells
0 hr	cells positive	48	62	9	2	121
	cells scored	161	230	38	23	452
	% positive	29.8	27.0	23.7	8.7	26.77
1 day	cells positive	58	80	26	18	182
	cells scored	209	216	42	34	501
	% positive	27.8	37.0	61.9	52.9	36.33
4 day	cells positive	137	86	18	9	250
	cells scored	218	173	28	19	438
	% positive	62.8	49.7	64.3	47.4	57.08

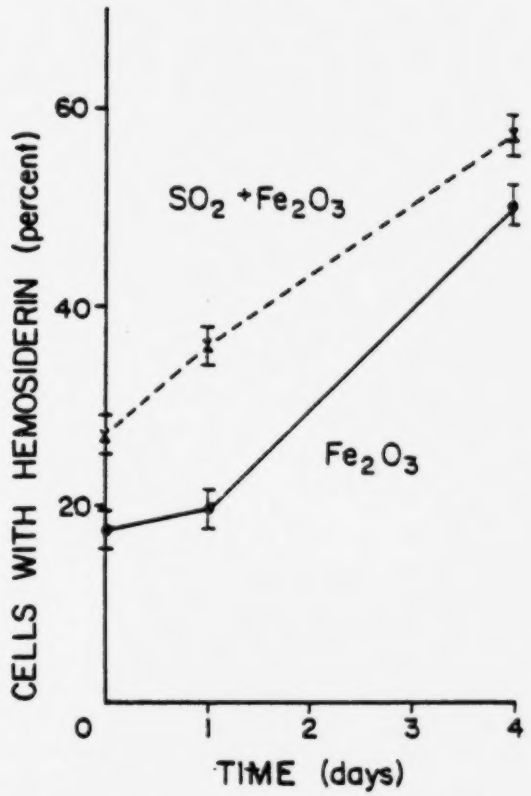

Figure 8 The mean percentage (± SE) of cell sections containing one or more hemosiderin granules versus time. Comparisons between Fe_2O_3 only (previous study, see text) and SO_2 plus Fe_2O_3 are shown.

Table 4 Activity of ^{99m}Tc in SO_2-exposed and nonexposed animals

	Animal	Lung weight (gm)	Counts/Minute (cpm)	cpm/g lung
Control	1	0.2268	706,000	3.11×10^6
	2	0.1768	657,000	3.72×10^6
	3	0.1800	547,000	3.04×10^6
	4	0.1812	879,000	4.85×10^6
	5	0.1936	528,000	2.73×10^6
				$\bar{x} = 3.49 \pm 0.84 \times 10^6$
SO_2	6	0.1913	717,000	3.75×10^6
	7	0.1597	393,000	2.46×10^6
	8	0.2107	557,000	2.64×10^6
	9	0.1762	498,000	2.83×10^6
	10	0.1892	647,000	3.42×10^6
				$\bar{x} = 3.02 \pm 0.54 \times 10^6$

in ($P < 0.01$). We also compared these superficial cells with the basal cells. In the 0-hr group, the basal cells had slightly fewer but not significantly less ($P > 0.1$) cell sections with hemosiderin. However, by 1 day, their participation rate was almost double that of the differentiated cells.

^{99m}Tc Retention

In order to determine if significant changes in ventilation or collection efficiency occurred following the SO_2 exposure, the activity/g lung was calculated for each animal after correction for the decay of the ^{99m}Tc ($T_{1/2}$ = 6.03 hr). Table 4 gives the weights and activities for the two groups of animals. Using Student's t test for comparing means, we found no statistically significant difference ($P > 0.2$) between the amount of sulfur colloid aerosol retained in the SO_2-exposed and nonexposed animals.

DISCUSSION

Particle Uptake

Gold and iron oxide were selected because they are small nontoxic particles that are readily endocytosed by phagocytic cells [18–21]. Although respiratory epithelial cells took up colloidal gold and iron oxide, the magnitude and perhaps mechanism of uptake differed greatly. Intracellular gold particles were seen infrequently in organelles (secondary lysosome, multivesicular body) that implied initial endocytotic ingestion. Secondary lysosomes have traditionally been considered the result of the fusion of phagosomes and primary lysosomes [22]. The life history of multivesicular bodies is poorly described. However, they are acid phosphatase positive and ingested material can be observed in them [23,24], so it is believed that they are also part of the lysosomal system. In addition, whatever little gold was taken up by the epithelium, it did not appear to be transported to the underlying connective tissue since no particles were observed in the submucosa.

By contrast, epithelial cells appear to have a greater affinity for iron than for gold colloid. Iron oxide is taken up from the airway lumen in pinocytotic vesicles. Although ferritin is found within multivesicular bodies and, by definition, hemosiderin is part of the lysosomal system, it cannot be concluded that the endocytotic uptake and intracellular pathways of iron oxide is similar to that of gold. There is evidence that endocytic vesicles may fuse directly with lysosomes or alternatively, they may be transported to the Golgi region and become incorporated at an earlier stage of the secretory or lysosomal system. Iron oxide [6], iron dextran [24], and cationic ferritin [25] have been seen within Golgi cisternea, GERL, or smooth endoplasmic elements and condensing vacuoles, as well as in multivesicular bodies and lysosomes. In addition, biochemical studies have shown that some of the apoferritin shells for ferritin are made in the rough endoplasmic reticulum, and the incorporation of iron to form ferritin occurs in the smooth endoplasmic reticulum [26,27]. Recent morphological evidence supports this concept [6,28].

The possibility also exists that iron oxide dissolves in the mucus and enters epithelial cells in an ionic form by a nonvesicular mechanism. Although in vitro preparations of the brush border from the intestine have been shown to bind Fe^{59}, the nature of this iron receptor is unknown [29,30]. Having entered the cell, iron may be taken up by apoferritin shells made by free ribosomes, it may cross endoplasmic membranes and be incorporated into apoferritin manufactured there [31,32] or it may enter into a labile pool of amino acid chelates [33].

We think that iron in the form of free ferritin in the cytosol or amino acid chelates is available for passage to basal cells and the submucosa.

In summary, although gold particles are not readily taken up, iron is transported by one and perhaps by two mechanisms. Once within cells, both particles enter the vacuolar lysosomal system but most likely at different entry points. Colloidal gold does not gain access to the submucosa, whereas iron, in some form, does.

SO_2-Induced Injury

The responses to SO_2 which we observed were similar to that seen in other studies using SO_2 or other noxious agents. Vacuoles appear as a reversible nonnecrotic change reflecting intracellular edema [34,35]. Vacuoles may also be part of an autophagocytic response; often contain cytoplasmic components, are positive for acid phosphatase, and upon recovery from an insult, assume the appearance of large cytoplasmic dense bodies [35,36]. Increased mucus has been reported in other studies using SO_2 [9–11], synthetic smog [37] and tobacco smoke [38]. The phenomena of polymorphonuclear leukocyte recruitment, although common with bacterial infections, has not been reported as a consistent response to airway insults. Other studies using SO_2 report an influx of leukocytes after chronic exposure [10,11] or after exposure in conjunction with carbon particles [9]. Our study is in agreement with other studies using SO_2 showing that the epithelium returned to normal within days to weeks after the exposure.

We wanted to see whether SO_2 initiated any changes in respiration that might persist during the particle exposure and hence affect the number of particles inhaled and retained by the animals. In our ^{99m}Tc aerosol experiment we compared the retention of this labeled aerosol in SO_2-exposed and nonexposed animals. We concluded that with our experimental design SO_2 had no significant effect on the amount of material retained. Likewise, Fig. 1 demonstrates that immediately after the colloidal gold exposure, extracellular particle clearance was similar in the two groups of animals.

SO_2 and Particle Uptake

In our system, SO_2 did not increase the rate of endocytic uptake of gold particles by bronchial epithelial cells. However, it was only in the SO_2-exposed animals that gold particles were found in the connective tissue compartment. The pathway by which these particles penetrated the epithelial barrier is not known. But it is of interest to note that gold in the submucosa was in the same section of tissue that particles within epithelial cells were found.

The response of the bronchial epithelium to iron uptake was affected by preexposure to SO_2. The percentage of cells participating in uptake was increased at each time point and the pattern of participation of the various cell types was different from that of the nonexposed animals. In our previous study [6], there was no difference between ciliated and nonciliated cells. Although in the SO_2-exposed animals there was initially no difference, by 1 day postexposure, the percentage of nonciliated cells with hemosiderin was greater, whereas by 4 days, the ciliated cells had a greater participation rate. Whether this observation reflects differences in the availability of iron oxide or differences in injury and recovery is not known.

We also noted that the pattern of response differed in the superficial layer versus the basal layer of epithelial cells. Without SO_2, the basal cells had signifi-

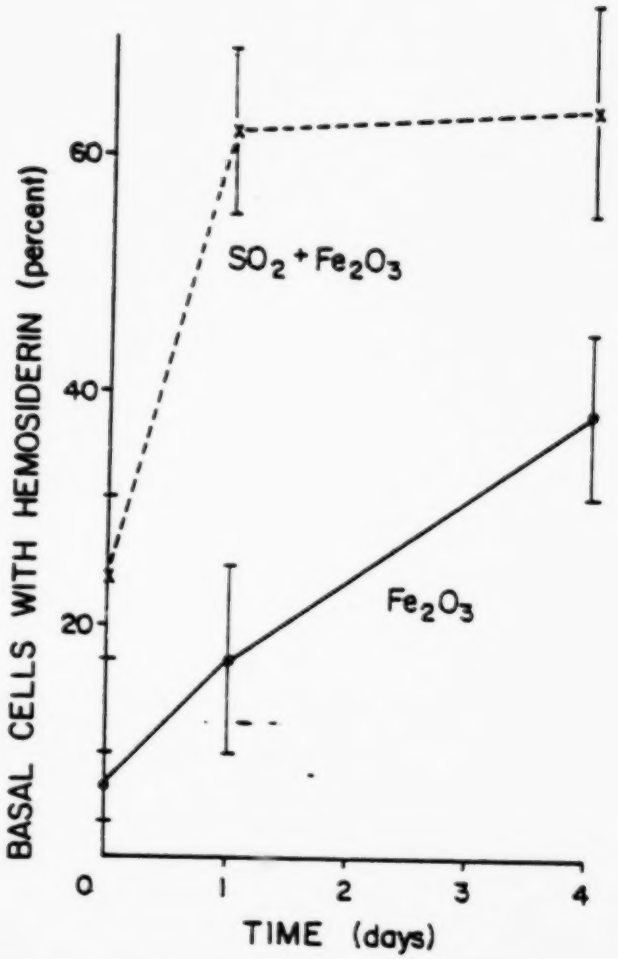

Figure 9 The mean percentage (± SE) of basal cell sections containing hemosiderin versus time. Comparisons between Fe_2O_3 only (previous study, see text) and SO_2 plus Fe_2O_3 are shown. In SO_2-exposed animals, the percentage is four times that of nonexposed animals at 0 hr and 1 day, and double at 4 days.

cantly less hemosiderin than the differentiated cells. Since the basal cells do not have access to luminal iron oxide we suggest that their source of iron for hemosiderin formation was chelates of iron or preformed ferritin from the superficial layer. However, in the SO_2 study, the percentage of basal cells with hemosiderin was no different from that of the differentiated cells in the 0-hr group and was significantly higher in the 1-day group. By 4 days, there was no statistically significant difference between the two layers but the basal cells still recorded the highest percentage. Basal cells from SO_2-exposed animals had a participation rate four times that of the nonexposed animals at 0 hr and 1 day and almost double their rate at 4 days (Fig. 9). When these dramatic increases in hemosiderin content of basal cells are coupled with an overall increase in iron uptake, it has

led us to the hypothesis that SO_2 exposure increases the nonvesicular transport of iron.

Whether increased iron uptake results from changes within an insulted epithelial cell or from changes affecting particle availability is not known. However, there is indirect evidence that factors affecting the particle may contribute to the increase. In studies conducted on iron absorption in the gastrointestinal tract, it has been found that iron solubility, hence its capacity to be absorbed, is sensitive to the pH of the luminal contents; as the acidity increases, the solubility increases [39]. The pH of the tracheobronchial mucus has been recorded to range from pH 6.5 to 7.5 [40]. SO_2 can react with the water in mucus to form sulfurous acid, which is weakly acidic. Another factor influencing the pH of mucus is its glycoprotein content. It has been shown that in conditions of increased mucous production such as cystic fibrosis [41] or chronic bronchitis [42], the amount of sulphated mucus increases. In the SO_2 study by Lamb and Reid [11], histochemical stains in rats revealed that after 4 days of SO_2 at 400 ppm for 3 hr/day, there was an increase in the acid glycoprotein content of the mucus. Whether the mucus in our SO_2 exposure had a change in its acidity or acid mucin content was not determined. However, an increase in uptake of iron due to greater availability could explain both the overall increase in epithelial hemosiderin content as well as the increases in basal cell hemosiderin.

In summary, we have shown that particle uptake and transport in bronchial epithelium from healthy mice varies, depending on the nature of the particle. Colloidal gold particles can be, but rarely are, pinocytosed by epithelial cells. Furthermore, no gold particles were seen in the underlying connective tissue compartment. Particulate iron oxide, however, is taken up by pinocytosis and perhaps solubilized iron enters by a nonvesicular mechanism. By exposing animals to SO_2, we produced a nonlethal insult to airway epithelial cells. Although no changes in the epithelial response to colloidal gold exposure were detectable, gold particles do, but infrequently, reach the submucosa by an unknown route. SO_2 exposure does increase uptake of iron by the epithelium. This increase may be due to an increase in the amount of soluble iron present in the mucus rather than a result of cell injury. We conclude that an insulted but intact epithelium is still reasonably effective in excluding particles. It also appears that properties of a particle are important in determining the response of injured as well as normal epithelium.

The writers thank Dr. Margaret Harbison, Steve LeMott, and Dr. Peter Valberg for assisting in the aerosol experiments, Bill Skornik for aiding in the SO_2 exposures, Dr. Henry Feldman for the statistical analysis, and Daryl Bichel, Susan Lite, and Leslie Phillips for typing the manuscript. This research was supported by Grant No. R80509103 from the Environmental Protection Agency and by Grant Nos. ES-01016 and ES-00583 from the National Institutes of Environmental Health Sciences.

REFERENCES

1. Richardson JB, Hogg JC, Bouchard T et al.: Localization of antigen in experimental bronchoconstriction in guinea pigs. J Allergy Clin Immunol 52:172–181, 1973.
2. Richardson JB, Bouchard T, Ferguson CC: Uptake and transport of exogenous proteins by respiratory epithelium. Lab Invest 35:307–314, 1976.
3. Mossman BT, Kessler JB, Ley BW et al.: Interaction of crocidolite asbestos with hamster respiratory mucosa in organ culture. Lab Invest 36:131–139, 1977.

4. Mossman BT, Adler KB, Craighead JE: Interaction of carbon particles with tracheal epithelium in organ culture. Envir Res 16:110–122, 1978.

5. Lauweryns JM, Baert JH: The role of pulmonary lymphatics in the defenses of the distal lung: Morphological and experimental studies of the transport mechanisms of intratracheally instilled particles. Ann NY Acad Sci 221:244–260, 1974.

6. Watson AY, Brain JD: Uptake of iron oxide aerosols by mouse airway epithelium. Lab Invest 40:450–459, 1979.

7. Simani AS, Inoue S, Hogg JC: Penetration of the respiratory epithelium of guinea pigs following exposure to cigarette smoke. Lab Invest 31:75–81, 1974.

8. Brain JD: The uptake of inhaled gases by the nose. Ann. Otol. Rhinol. Laryngol. 79: 529–539, 1970.

9. Asmundsson T, Kilburn K, McKenzie W: Injury and metaplasia of airway cells due to SO_2. Lab Invest 29:41–53, 1973.

10. Johnson HD, Lincoln EM, Flatt RE: SO_2 exposure and recovery effects on mice. Proc Soc Exp Biol Med 139:861–864, 1972.

11. Lamb D, Reid L: Mitotic rates, goblet cell increase and histochemistry changes in mucus in rat bronchus epithelium during exposure to SO_2. J Path Bact 96:97–111, 1968.

12. Brian JD, Valberg PA, Sorokin SP et al.: An iron oxide aerosol suitable for animal exposures. Environ Res 7:13–26, 1974.

13. Valberg PA, Brain JD: Generation and use of three types of submicronic iron-oxide aerosol. Amer Rev Resp Dis 120:1013–1024, 1979.

14. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137A, 1965.

15. Karnovsky MJ: Use of ferrocyanide-reduced OsO_4 in electron microscopy. No. 284. Eleventh Annual Society for Cell Biology, New Orleans LA, p 146, 1971.

16. Ainsworth SK, Karnovsky MJ: An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thin sections. J Histochem Cytochem 20: 225–229, 1972.

17. Munro HN, Linder MC: Ferritin: structure, biosynthesis and role in iron metabolism. Physiol Rev 58:317–396, 1978.

18. Cohen ZA, Fedorko ME, Hirsch JG: The in vitro differentiation of mononuclear phagocytes. V. The formation of the macrophage lysosome. J Exp Med 123:757–766, 1966.

19. Kavet RI, Brain JD: Phagocytosis: quantification of rates and intercellular heterogeneity. J Appl Physiol: Resp. Environ Exercise Physiol 42:432–437, 1977.

20. Sargent KS, Munro HN: Association of ferritin with liver cell membrane fractions. Exp Cell Res 93:15–22, 1975.

21. Grant MM, Sorokin SP, Brain JD: Lysosomal enzyme activities in pulmonary macrophages from rabbits breathing iron oxide. Am Rev Resp Dis 120:1003–1012, 1979.

22. Straus EW: Cytochemical observations on the relationship between lysosomes and phagosomes in kidney and liver by combined staining for acid phosphatase and intravenously injected horseradish peroxidase. J Cell Biol 20:497–507, 1964.

23. Friend DS, Farquhar MG: Functions of coated vesicles during protein absorption in the rat vas deferens. J Cell Biol 35:357–376, 1967.

24. Herzog, V, Farquhar MG: Luminal membrane retrived after exocytosis reaches most Golgi cisternea in secretory cells. Proc Natl Acad Sci 74:5073–5077, 1977.

25. Farquhar MG: Recovery of surface membrane in anterior pituitary cells: variations in traffic detected with anionic and cationic ferritin. J Cell Biol 77:R35–42, 1978.

26. Lee SSC, Richter GW: Biosynthesis of ferritin in rat cells and rat livers. I. Synthesis and assembly of protein subunits of ferritin. J Biol Chem 252:2046–2053, 1977.

27. Lee SSC, Richter GW: Biosynthesis of ferritin in rat cells and rat livers. II. Binding of iron by ferritin protein. J Biol Chem 252:2054–2059, 1977.

28. Lee JCK, Lee SS, Schlesinger KJ et al.: Production of ferritin by rat hepatoma cells *in vitro*. Demonstration of protein subunits and ferritin by immunofluorescence. Am J Pathol 80:235–248, 1975.

29. Greenberger NJ, Balcerzak SP, Ackerman G. A.: Iron uptake by isolated intestinal brush border: changes induced by alterations in iron stores. J Lab Clin Med 73: 711–721, 1969.

30. Hubers H, Hubers E, Forth W et al.: Binding of iron and other metals in brush borders of jejunum and ileum of the rat in vitro. Acta Pharm Toxicol 29 (Supp. 4):22, 1971 (abst).

31. Puro DG, Richter GW. Ferritin synthesis by free and membrane-bound polyribosomes of rat liver. Proc Soc Exp Biol Med 138:399–403, 1971.

32. Redman CM: Biosynthesis of serum proteins and ferritin by free and attached ribosomes of rat liver. J Biol Chem 244:4308–4315, 1969.

33. Linder MC, Munro HN: The mechanism of iron absorption and its regulation. Fed Proc 36:2017–2023, 1977.

34. Trowell OH: The experimental production of watery vacuolation of the liver. J Physiol 105:268–297, 1946.

35. Trump BF, Janigan DJ: The pathogenesis of cytologic vacuolization in sucrose nephrosis. Lab Invest 11:395–411, 1962.

36. Fedorko ME, Hirsch JG, Çohen ZH: Autophagic vacuoles produced *in vitro*. I. studies on cultured macrophages exposed to chloroquine. J Cell Biol 38:377–391, 1968.

37. Tremer HM, Falk HL, Kotin P: Effect of air pollutants on ciliated mucus-secreting epithelium. J Natl Cancer Inst 23:979–997, 1959.

38. Lamb D, Reid L: Goblet cell increase in rat bronchial epithelium after exposure to cigarette and cigar tobacco smoke. Brit Med J 1:33–35, 1969.

39. Jacobs A, Miles PM: Role of gastric secretion in iron absorption. Gut 10:226–229, 1969.

40. Holmer B, Lindergren M, Anderson JM: pH effects on ciliomotility and morphology of respiratory mucosa. Arch Envir Health 32:216–226, 1977.

41. Lev R, Spicer S: A histochemical comparison of human epithelial mucins in normal and in hypersecretory states including pancreatic cystic fibrosis. Amer J Path 46: 23–47, 1965.

42. DeHuller R, Reid L: Adult chronic bronchitis. Medicina Thoracalis 22:549–567, 1965.